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ANNUAL REPORT FOR AWARD DAMD17-98-1-8624

REVERSAL OF MITOCHONDRIAL DAMAGE CAUSED BY ENVIRONMENTAL TOXINS

Principal Investigator: Martin R. Gluck MD PhD

INTRODUCTION- The present grant entitled "Reversal of Mitochondrial Damage Caused by Environmental Toxins" was originally assigned to Dr. Gerald Cohen PhD, for the funding period of January 1998-December 2002. Regretfully, Dr. Cohen passed away following a prolonged chronic illness in 2001, and the grant was subsequently assigned to Dr. Catherine Mytilineau PhD for the subsequent eight months in 2001. Around January 2002, I requested and was approved to become the principal investigator for the completion of the grant. As a condition for completion of the studies I requested an extension, which I was granted, so that the present grant will run until October 2003. The annual report will initially summarize the findings of Drs. Cohen and Mytilineau, and conclude with my work..

II. **WORK ACCOMPLISHED: Dr G. Cohen:** The original intent of the grant, as proposed by the principle investigator, Dr. G. Cohen, was to examine the ability of monoamine oxidase-associated substrates such as dopamine and tyramine to inhibit mitochondrial respiration. The studies entailed initially characterizing the ability of the two substrates to inhibit respiration in brain mitochondria, and to subsequently identify and characterize the underlying biochemical mechanisms responsible for the inhibition. Dr. Cohen proposed the possibility that monoamine oxidase (MAO)-derived production of hydrogen peroxide could hypothetically lead to oxidative damage within the mitochondria, and induce mitochondrial dysfunction. In one publication he reported that the inhibition of mitochondrial respiration by MAO substrates was associated with significant increases in levels of glutathione-protein-mixed disulfides, and that covalent modification of protein thiols by glutathione could inactivate critical thiol-dependent proteins within the electron transport chain (Cohen and Kessler, 1999).

B) These findings have potential importance in understanding mechanisms underlying processes of neurodegeneration. Dr. Cohen believed that these findings could have direct relevance to the investigation of mechanisms underlying mitochondrial dysfunction following exposure to environmental neurotoxins as well as contributing to the development and progression of Parkinson's Disease. The central link between the different forms of neurodegeneration was that oxidative stress played a critical role in the degenerative pathway, and that monoamine oxidase-mediated turnover of substrates was the source of reactive oxygen species leading to inhibition of respiration.

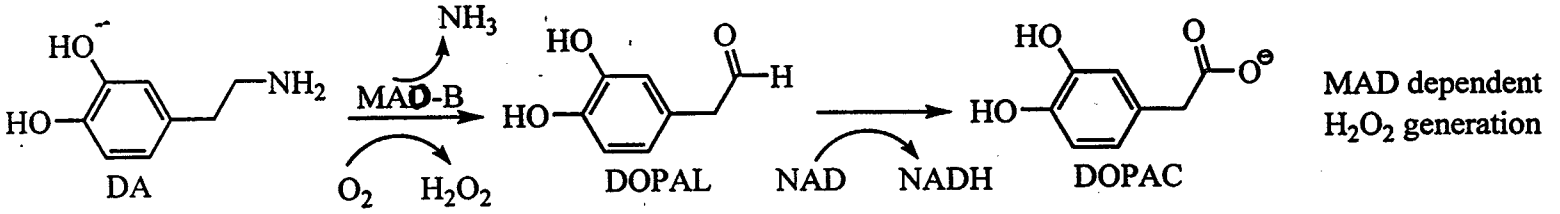
Dr. Cohen's primary focus of oxidative stress pathways was that hydrogen peroxide generation by MAO led to oxidation of glutathione (GSH) to oxidized glutathione disulfide (GSSG). The glutathione disulfide was then able to glutathionylate protein cysteinyl thiols in proteins (Scheme 1d). Covalent modification of protein thiols by GSSG could in principle inactivate enzyme activity of proteins in the electron transport chain and account for the observed adverse effects on mitochondrial function following exposure to dopamine or tyramine.

C) Dr. Cohen proposed to identify the glutathione-modified mitochondrial proteins formed following exposure to dopamine using protein gel electrophoresis on radiolabeled glutathione-modified proteins. He also proposed to determine how modification of experimental conditions could prevent or reverse the inhibition of brain synaptic or non-synaptic mitochondrial respiration.

III. **WORK ACCOMPLISHED: Dr. C. Mytilineau**

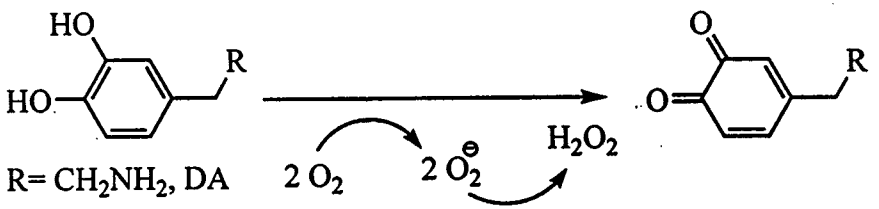
A) Dr. Mytilineau has enormous expertise and longstanding credentials in investigations examining the role of GSH depletion in the development of neurotoxicity. She has used

SCHEME 1a



MAD dependent
H_2O_2 generation

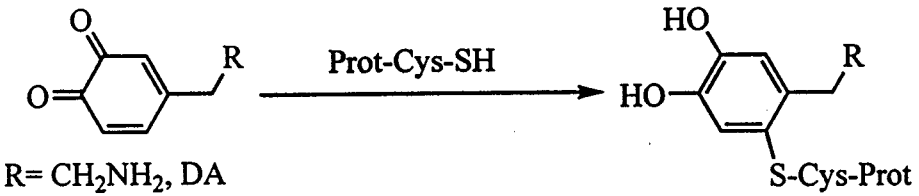
SCHEME 1b



Non MAO
H_2O_2 generation

R= CH_2NH_2, DA
R= COH, DOPAL
R= CO_2^-, DOPAC

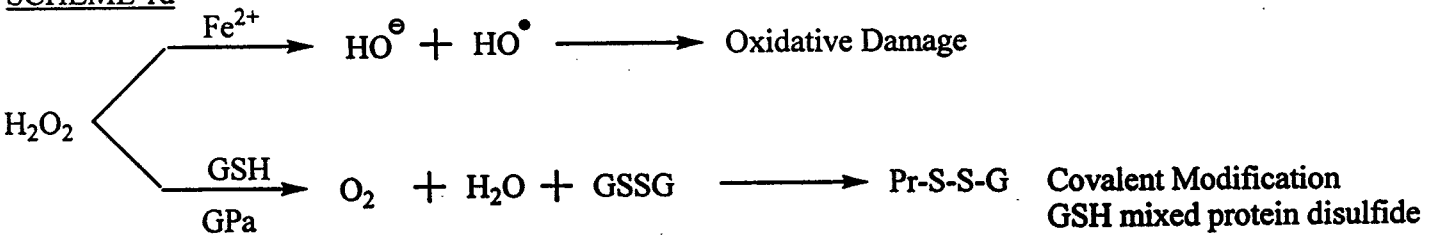
SCHEME 1c



Thiol modification of
Cys-DOPA-Quinone

R= CH_2NH_2, DA
R= COH, DOPAL
R= CO_2^-, DOPAC

SCHEME 1d



Covalent Modification
GSH mixed protein disulfide

mesencephalic dopaminergic mixed-cell culture models to examine the effects of GSH depletion on the development of neurotoxicity. Although Dr. Mytilineau was PI for a very short period of time, and could not pursue Dr. Cohen's experimental paradigms, she contributed significantly to identifying a potential novel neuroprotective mechanism invoked following exposure to oxidative stress, and which may have significant relevance to the aims proposed in Dr. Cohen's original proposal.

In her studies Dr. Mytlineau examined the effects of lipopolysaccharide (LPS) exposure on the induction of neurotoxicity in GSH-depleted cell culture (Kramer et al. 2002). The results of the studies showed that LPS exposure increased levels of Mn-superoxide dismutase (Mn-SOD) in LPS treated cells, indicating that increases in this antioxidant enzyme were likely occurring in response to the development of oxidative stress. *These findings are potentially relevant to aims proposed in the present grant, and studies are proposed to carry out parallel studies in cell cultures exposed to dopamine.*

IV. WORK ACCOMPLISHED: Dr. M. Gluck (Present PI)

A) *DA is able to inhibit mitochondrial respiration in isolated rat brain mitochondria.* Addition of DA to isolated rat brain mitochondria leads to a dose- and time-dependent inhibition of mitochondrial respiration. The DA dose-response curve for inhibition of pyruvate-malate supported respiration (mitochondrial ETC complexes I-III-IV) after fifteen-minute DA incubations at 30°C demonstrates that millimolar concentrations of DA are required to obtain measurable amounts of inhibition (Figure 1). The Ic50 of the inhibition is approximately 16 mM, indicating that in well-coupled intact mitochondria and the indicated assay conditions DA is a relatively poor inhibitor of respiration. However, dopamine's inhibitory potency appears to be dependent on the mitochondrial coupling integrity (as

measured by respiratory control ratios- state 3 / state 4 respiratory activities). Table 1 shows a comparison of the relative effects of DA concentrations on the inhibition of respiration in mitochondria with varying respiratory control ratios. *It appears that as mitochondria become increasingly uncoupled their sensitivity to inhibition of respiration by DA increases. These findings suggest that conditions that reduce the coupling of mitochondrial oxidative phosphorylation may lead to increases in dopamine's potential to inhibit respiration.*

B) *Effect of DA turnover by MAO on pyruvate-malate respiration in rat brain mitochondria.*

Figure 1 also shows the effects of MAO inhibition on the inhibition of respiration by DA. In these experiments inhibition of MAO-A & B was accomplished using pargyline/chlorgyline (4 μM) co-additions. As seen in Figure 1, at relatively low concentrations of DA (<30% inhibition of respiration) inhibition is completely reversible in the presence of MAO inhibitors. At higher DA concentrations (>30% inhibition of respiration) there appears a MAO-independent inhibitory pathway which accounts for about 25%-35% of the total inhibitory contribution. *These results show that throughout the DA dose-response curve the majority of inhibition of respiration arises from MAO mediated oxidation of DA, but at higher DA concentrations MAO-independent mechanisms become important in the overall inhibitory process. This indicates that the inhibition of respiration by DA arises from DA turnover products of MAO metabolism (low [DA]), and to a lesser degree chemical breakdown turnover products of DA in the absence of MAO metabolism (high [DA]). These observations implicate the following candidate compounds as potential mediators of respiratory inhibition (scheme 1); 1) H_2O_2 or HO 2) NH_3 3) DOPAL 4) DA-quinones 5) GSSG [via protein thiol-glutathioine mixed disulfide (Pr-S-SG) formation].*

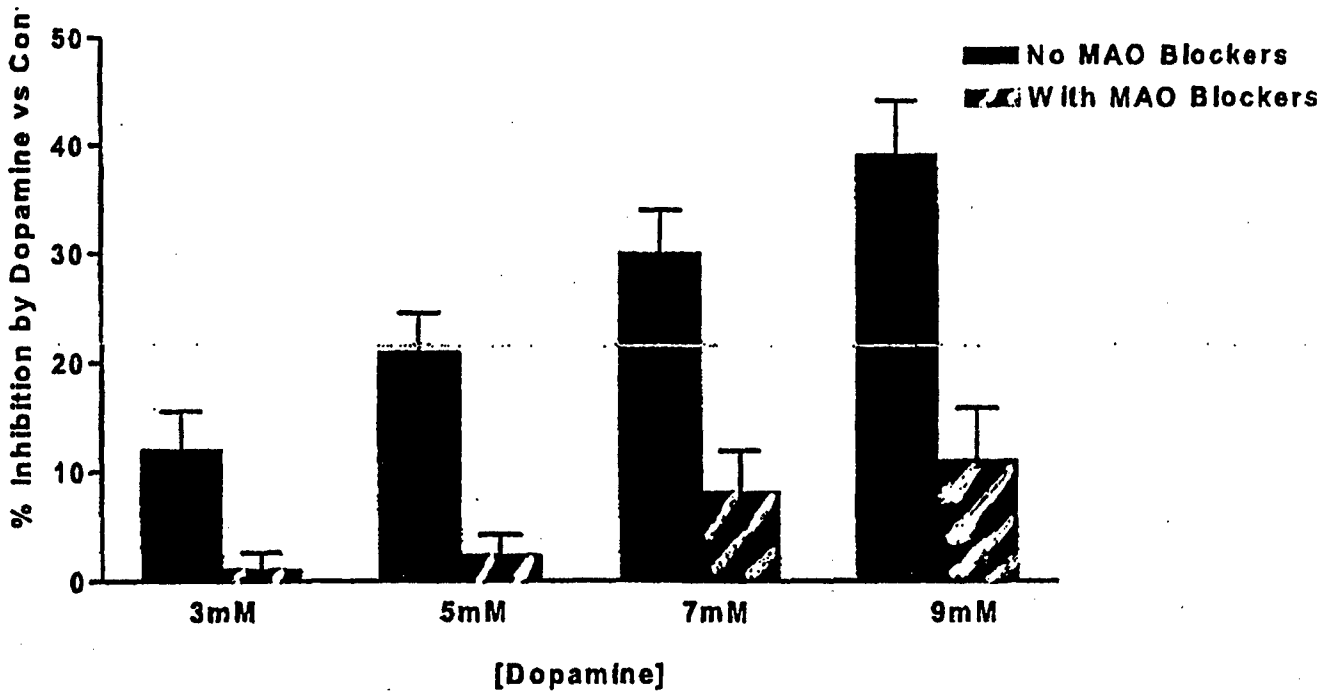


Figure 1. Rat brain mitochondria were treated with varying concentrations of dopamine ± pargyline/chlogyline (4 μM final conc.) for 15 min at 30°C, % inhibition of respiration by dopamine is reported as mean ± SD. n is from separate determinations from three mitochondrial preparations. It can be readily observed that millimolar concentrations of dopamine (Ic50 = 16.5 mM + 5.6, n=3) are required to inhibit pyruvate/malate-stimulated respiration in well-coupled mitochondria (RCRs ≥ 8). At dopamine concentrations producing less than 25%-30% inhibition there is nearly complete reversibility in the presence of MAO inhibitors. At dopamine concentrations producing greater than 30% inhibition ([DA] ≥ 7mM) there is a minor but significant component of the inhibition which is not reversible in the presence of MAO inhibitors.

Table 1. Comparison of dopamine potency in inhibiting brain mitochondrial respiration as a function of respiratory control ratios (state 3/state 4). Respiratory measurements were obtained using a Clark oxygen electrode in the presence of malate/pyruvate (5 mM each) following a 15 minute pre-incubation in the presence of 5 mM dopamine, followed by addition of ADP (0.50 mM).

[Dopamine] mM	RCR values	% inhibition of respiration
5 mM	3-5	40%
5 mM	6-8	20%
5 mM	9-10	<10%

C) Comparison of inhibitory potencies of H2O2 and GSSG on pyruvate-malate supported respiration in brain mitochondria: Putative identification of H_2O_2 as a causative agent of respiratory inhibition. Table 2 shows a comparison of the effects of DA, H_2O_2 and GSSG exposure on pyruvate-malate supported respiration in brain mitochondria. These results demonstrate that exposure to DA or H_2O_2 leads to respiratory inhibition, but no such effect is seen with GSSG. Table 3 shows that upon the separate addition of 1mM GSSG there are corresponding elevations of Pr-S-S-G. The largest increase was observed with 1mM GSSG preincubations (3-4-fold increases in both the matrix space and intermembrane fractions), however, these increases did not correlate with any measurable inhibition of respiration. To further confirm that the lack of inhibition by GSSG was due to inadequate formation of Pr-S-S-G within the ETC we exposed GSSG to freeze-thawed lysed mitochondria, where the ETC can come in direct contact with the GSSG. As with mitochondria, no observable inhibition of any ETC complex was measured in the presence of very high concentrations of GSSG. *These results indicate that the origin of respiratory inhibition by DA is unlikely a result of increased Pr-S-S-G formation within the ETC due to GSSG formation following MAO-mediated oxidation of DA* (Gluck et al 2002).

In contrast to GSSG exposure, H_2O_2 exposure leads to significant inhibition of respiration in intact brain mitochondria (Table 2 and Figure 2). Inhibitory dose-response curves for H_2O_2 demonstrates significant inhibition of respiration in the micromolar range. For ETC-complex I-associated respiration Ic50 values range between 100μM-200μM, whereas Ic50 values for inhibition of Complex II- mediated succinate oxidation is in the range of 50μM-100μM. Similar results were observed in freeze-thawed mitochondrial lysates (Figure 2). To determine whether H_2O_2 played a direct role in the inactivation of the ETC complexes the iron chelator

Table 2. Inhibition of mitochondrial respiration does not correlate with an increase in mitochondrial glutathione–protein–mixed disulfides

Condition	GSH–protein–mixed disulfide		O_2 consumption	
	n	(-fold change ± SEM)	*n*	(% control ± SEM)
Control	12	1.00 ± 0.07	12	100 ± 6.1
100 μM H_2O_2	6	1.60 ± 0.15[a]	6	56 ± 3.3[a]
500 μM DA	3	1.53 ± 0.19[a]	3	78 ± 2.6[a]
1 mM GSSG	3	3.30 ± 0.57[a]	3	108 ± 4.4

Rat brain mitochondria were isolated as described in Methods and incubated with either dopamine (DA), H_2O_2 or oxidized glutathione (GSSG) at the above indicated concentrations for 30 min at 30°C. At the end of incubation, mitochondria were analyzed for glutathione–protein–mixed disulfide formation. In separate incubations, from the same preparations, mitochondria were exposed to the above compounds using incubation conditions as described in the legends to Figs 2 and 3 and the effects on pyruvate/glutamate/malate supported respiration was determined. [a]Different from control, $p < 0.05$.

Table 3. Effect of extramitochondrial GSSG on intramitochondrial GSH, GSSG and glutathione–protein–mixed disulfides (nmol/mg protein ± SEM)

Condition	Intra-mito GSH	Intra-mito GSSG	GSSG/ GSH	Membrane GS~S~prot.	Matrix GS~S~prot
Control	3.01 ± 0.50	0.13 ± 0.06	0.043	0.68 ± 0.08	0.16 ± 0.03
1 mM GSSG	13.40 ± 2.10[a]	3.41 ± 0.90[a]	0.254	1.43 ± 0.24[a]	0.57 ± 0.19[a]

Isolated rat brain mitochondria were incubated in the presence or absence of 1 mM GSSG for 30 min at 30°C. At the end of incubation, the mitochondria were washed, lysed and the intramatrix GSH and GSSG content were measured by HPLC. Glutathione–protein–mixed disulfide formation was measured separately in soluble matrix proteins and in the total mitochondrial membrane fraction. *n* is from four to six determinations per condition. [a]Different from control, $p < 0.05$.

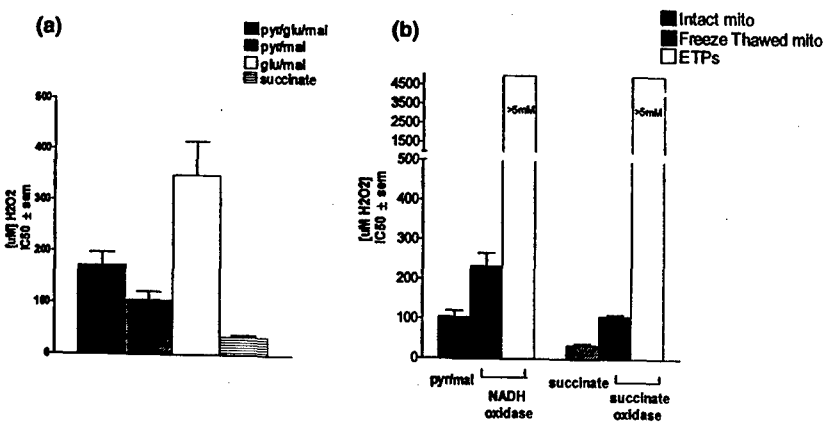


Fig. 2. (a) Rat brain mitochondria were treated with different concentrations of H_2O_2 (10–1000 μM) for 5 min, 30°C and the rate of O_2 consumption using different substrates to support respiration was determined. IC_{50} were calculated from nonlinear regression plots (GraphPad, Inplot). *n* is from three to five dose–response determinations per substrate. (b) Intact mitochondria, freeze–thawed mitochondria or electron transport particles (ETPs) were exposed to different concentrations of H_2O_2 for 5 min and the effects on O_2 consumption for pyruvate/malate- or succinate-supported respiration (intact), or NADH oxidase or succinate oxidase activities (freeze–thawed or ETPs) were determined as described in Methods. *n* is from three to four determinations per dose–response for intact and freeze–thawed mitochondria and two for ETPs.

desferroxamine was co-administered with the H_2O_2 in freeze-thawed lysed mitochondria (Figure 3). Desferroxamine was able to significantly attenuate the inhibitory effects of H_2O_2 indicating that $HO^{\cdot}$ is likely to be responsible in part for the underlying respiratory inhibition. Similar studies could not be performed in intact mitochondria because desferroxamine does not diffuse across the IMM into the matrix. *These cumulative results indicate that in contrast to GSSG, H_2O_2 appears to be responsible for respiratory inhibition. Furthermore, hydroxyl radical formation, resulting from Fenton- or Haber-Weiss chemistry, is likely to be an important mediator of the inhibition of mitochondrial respiration and ETC function.*

D. *Comparison of the potencies of DA in inhibiting mitochondrial respiration versus ETC activities in freeze-thawed lysates.*

a) Figure 4 shows the effects of DA exposure on ETC activities in freeze-thawed lysed mitochondria. In contrast to intact mitochondria where the Ic50 for DA-induced inhibition is 16 mM, the Ic50 for inhibition of ETC complexes I-III-IV (NADH oxidase), I-III (NADH-cyt C reductase) and II/III (succinate-cyt C reductase) by DA is nearly 100-fold lower (ranges 100μM-250μM). Furthermore, the inhibition could not be reversed in the presence of pargyline/chlorgyline co-additions, or when 500 μM of the non-catechol containing MAO-substrate tyramine was used instead of DA (data not shown). *Therefore, DA is a much more potent inhibitor of ETC function when it can gain direct access to the ETC. Secondly, the lack of any effect by MAO inhibitors or tyramine on the inhibition implies that the inhibitory mechanism may involve MAO-independent H_2O_2 formation or covalent modification of proteins via DA-quinone formation (Scheme 1c & 1d).*

b) To determine whether it was possible to increase the permeability of DA across the IMM of well-coupled mitochondria (RCRs > 9) we co-incubated DA with the lipophilic anion

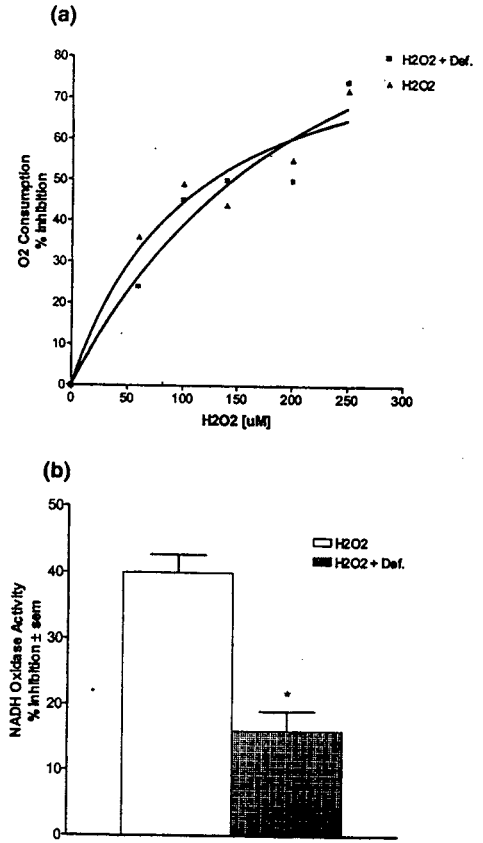


Fig. 3 (a) Brain mitochondria were exposed to different concentrations of H_2O_2 in the presence or absence of 1 mM desferrioxamine for 5 min at 30°C and the effects on pyruvate/glutamate/malate supported respiration was determined. (b) Rat brain mitochondria were lysed by freeze–thawing and exposed to sufficient H_2O_2 for 5 min at room temperature to produce approximately 50% inhibition of NADH oxidase activity (400–800 μM). Parallel incubations were carried out in the presence of 1 mM desferrioxamine. NADH oxidase activity was measured spectrophotometrically by following the oxidation of NADH to NAD in a dual beam spectrophotometer. *n* is from three determinations. *Different from H_2O_2 alone.

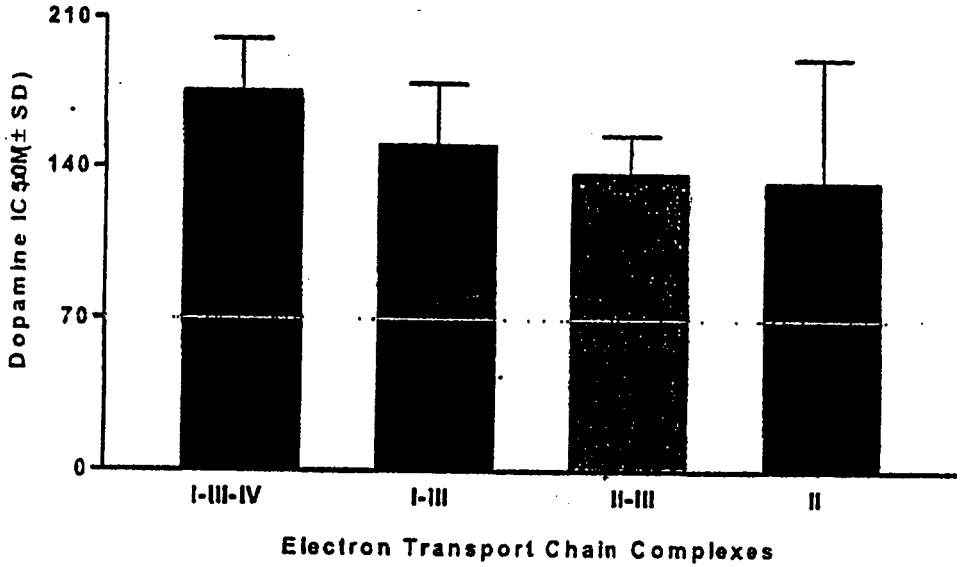


Figure 4 Dopamine is a Potent Inhibitor of Electron Transport Chain Complexes in Lysed Mitochondria

Freeze-thawed brain mitochondria were assayed spectrophotometrically for NADH oxidase activity (combined complex I/III/IV), cytochrome c reductase (combined complex I/III), succinate-cytochrome c reductase (combined complex II/III) and succinate-CoQ_1 reductase (Complex II) following a 15 min incubation with dopamine at 25°C. IC50 values are reported as mean + SD. n is from three determinations of separate mitochondrial preparations. Similar to that observed with H2O2 exposure, ETC complex activities are significantly decreased in the presence of dopamine. The Ic50 values for dopamine inhibition are also similar to that observed with H2O2: combined complexes I-III-IV (175μM ± 25μM, n=3), I-III (150μM ± 29μM, n=4), II-III (137μM ± 18μM, n=3) and II (133μM ± 58μM, n=3).

tetraphenyl boron (TPB^-), a compound which has been previously found to potentiate the inhibition of respiration by related ammonium compounds including MPP^+ and methamphetamine. TPB^- potentiated MPP^+ inhibition 10-fold and methamphetamine 10-fold, however, no potentiation was observed when TPB^- was added to DA (data not shown). *These results indicate two important findings; first, the inner mitochondrial membrane provides a very effective barrier preventing DA diffusion into the matrix space, protecting the electron transport chain against the potent inhibitory effects of DA. Secondly, mitochondrial respiration and electron transport chain function is potentially vulnerable to DA-associated inhibition either by enzymatic MAO-mediated pathways or MAO-independent pathways which together are likely to involve the formation of ROS and DA-quinones.*

WORK PROPOSED

The work proposed in the following remaining months of the grant will be aimed at further characterizing the biochemical mechanisms underlying inhibition of mitochondrial respiration by DA, and identifying the molecular targets within the mitochondria responsible for the inhibition. The following experiments are proposed:

A) To perform parallel time- and dose-response respiratory inhibition studies with non-catechol containing MAO-metabolized substrates (i.e. tyramine or 3-O-methyldopamine) to confirm that non-MAO mediated inhibition of respiration at high concentrations of DA derive from reactions at the catechol ring. If catechol oxidation underlies inhibition of respiration at high [DA], then no such inhibitory component should be observed using tyramine or 3-O-methyldopa. Similarly, catechol containing non-MAO substrates such as DOPAL OR DOPAC provide a measure of the catechol moiety potency for inhibiting respiration in the absence of MAO-associated oxidation.

respiration or ETC activities will be stimulated in the presence of malate/glutamate (mitochondria) or NADH or succinate (freeze-thawed).

F) The last experiments are aimed at identifying whether exposure of dopamine in mesencephalic mixed-dopamine cell cultures leads to increased expression of Mn-SOD, as measured by Western blotting techniques.

KEY RESEARCH OUTCOMES

Our research demonstrated the following facts related to the cause of induction of brain mitochondrial dysfunction related to dopamine metabolism by MAO.

1) Dopamine oxidation by MAO leads to time and dose-dependent inhibition of brain mitochondrial respiration. The potency of dopamine in inhibiting respiration is dependent on the integrity of the mitochondria (range of IC50 values are 5 mM – 16 mm).

2) With the initial 30%-35% inhibition of mitochondrial respiration cessation of respiration derives exclusively from MAO-associated metabolism of dopamine. At greater than 35% inhibition the inhibition becomes partially independent of MAO activity. These findings suggest that dopamine is capable of inhibiting respiration by MAO-dependent and MAO-independent mechanisms. However, the two pathways may have the common feature of generating reactive oxygen species leading to oxidative damage of the mitochondria.

3) Dopamine is significantly more toxic to respiratory function when it diffuses across the inner mitochondrial membrane into the matrix space. Dopamine is capable of inhibiting Complex I and Complex II activities in freeze-thawed mitochondria, and is nearly 100-fold more potent than in intact mitochondria. These findings demonstrate that conditions that increase the permeability of dopamine across the inner mitochondrial membrane can lead to significant potentiation of inhibition.

4) The underlying mechanisms responsible for inhibition of respiration and electron transport chain activities is likely related to reactive oxygen species generation and oxidative stress. In contrast to hypotheses forwarded originally by Dr. Cohen, our data indicates that induction of mitochondrial dysfunction by dopamine is not a result of glutathione-protein-mixed disulfide formation, but rather, from production of hydrogen peroxide (MAO-mediated) and hydrogen peroxide and superoxide formation (non-MAO mediated pathways).

REPORTABLE OUTCOMES

Zeevalk, GD, Erhart J. and Gluck M. Dopamine metabolism and effects on brain mitochondrial respiration. Society of Neuroscience, 2001, San Diego.

Gluck MR, Gauptam A, Cheong J, Mattice L and Zeevalk G. Inhibition of mitochondrial respiration by dopamine primarily arises from monoamine oxidase-mediated oxidation. Society of Neuroscience, 2002, Orlando.

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CONCLUSIONS

Dopamine is potentially neurotoxic to brain cells, by mechanisms that may be mediated by MAO-linked oxidative stress. Mitochondrial dysfunction can be induced by dopamine metabolism by MAO, and therefore cells exposed to high levels of dopamine or related MAO substrates may be at increased risk of cell injury and death. The pathways leading to mitochondrial dysfunction appear to most associated with the formation of reactive oxygen species, and may not be related to formation of glutathione-protein mixed disulfides.

Furthermore, dopamine can inhibit electron transport chain function in the absence of MAO-mediated oxidation. Our findings suggest that dopamine is capable of inducing mitochondrial dysfunction through enzymatic and non-enzymatic mediated pathways, although both are appear to involve induction of oxidative stress. These findings suggest an ongoing role for antioxidant therapy or inhibition of MAO as a therapeutic intervention for the treatment of Parkinson's Disease, or from protection from neurotoxic substrates metabolized by MAO in the central nervous system, such as agents that our armed forces may be exposed to.

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